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ADDENDA

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Addendum 1

COMPREHENSIVE REPORT ON THE ACHIEVEMENTS OF THE "GROUP"

Historical:

The origin of the group goes back to the summer of 1972 when Paul Hamosh transferred from the Veterans Administration to Georgetown and got together with Dr. Vidic to establish a group dedicated to "Structure and Function of the Lung." In the Fall of 1972, Dr. Bellanti contacted Dr. Hamosh and informed him about his intentions to apply for a Program Project in Lung Immunology and invited him to participate. Dr. Hamosh also recommended Dr. Vidic, as a participant, and a basis for close co-operation was established.

However, not all activities were immediately integrated. In January 1973, Dr. Hamosh became the recipient of an award from the Center for Tobacco Research for the study of "Smoking and the Small Airways." This study was a continuation of research performed by Dr. Hamosh and Dr. Da Silva with the support of the Veterans Administration (Dr. Da Silva was a fellow in pulmonary medicine at the VA hospital at that time).

It became soon clear, that without a biochemist, the research on structure and function has no depth. Application was made for a second Center grant: "Smoking and Lung Metabolism" in which provisions were made for hiring a biochemist. The proposal was funded 23 January 1974 and by July 1974 we succeeded in recruiting Dr. Margit Hamosh who spent the previous nine years at the National Institutes of Health. We were also lucky in acquiring the services of Dr. Schechter, a biochemist with considerable experience who, unfortunately for us, will return to Israel in the near future.

In the meantime, even before funding by CTR, the group started working together with the aid of the Washington Heart Association. Most of this work was centered around the incorporation of radio-labeled palmitate into isolated perfused lungs as a function of ventilation. During the early stages of research in 1974, it became clear that biochemistry of tissue slices or homogenates or even isolated perfused lungs might yield answers to complex problems requiring pure populations of cells. Indeed, the arrival of Dr. Jean Wrathall in January 1975 was greeted with great enthusiasm. We gained a tissue culture expert of excellent background (Cornell University) and experience. At about the same time, we discovered that Dr. Goeringer, who just returned from a sabbatical leave at Oxford, has extensive experience in cell separation techniques and organ cultures. The addition of these two people to the group rounded and expanded the scope of our activities.

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Another significant development was the realization that with the expanded workload, Dr. P. Hamosh could not perform his research in physiology without significant input by an additional skilled investigator. Fortunately for the group, Dr. Da Silva, who spent two years in Portugal, agreed to join us, which he did in the Fall of 1974. The most recent addition to the group, Dr. Rabinowitz came to us when we started work on human lung tissues. It became clear, that without the cooperation of a pathologist, such a project will not get off the ground. Thanks to him , we have now a steady flow of specimens.. He is also interested in directing our effort in morphometry of the human lung.

The group is now a well rounded one and further additions will be on the "junior" level. We envision a number of post-doctoral fellows working under the supervision of the investigators. The program project will now provide the backbone of this organization.

The Pulmonary Program Project, the one directed by Dr. Bellanti, is centered around the role of the macrophage in the defense of the lung. It provides, in addition to cooperation in sharing resources (technical help and specimens), a contact point through project #4 (Maturation Studies of Cellular and Immunological Processes in Pulmonary Defense Systesm). Drs. Vidic and M. Hamosh are active co-investigators in that project..

The group has considerable outside support. Most significant are the computer support of the National Biomedical Research Foundations and within it a Princeton educated computer expert Dr. Robert Shile. Dr. P. Hamosh is still supported by the Veterans Administration. Excellent collaborative arrangements exist between different laboratories at the National Institutes of Health and the group (in NHLI, NIAMDD and NICHD). The following scheme indicates the historical development of the group and some of its present intra and extra-mural activities.

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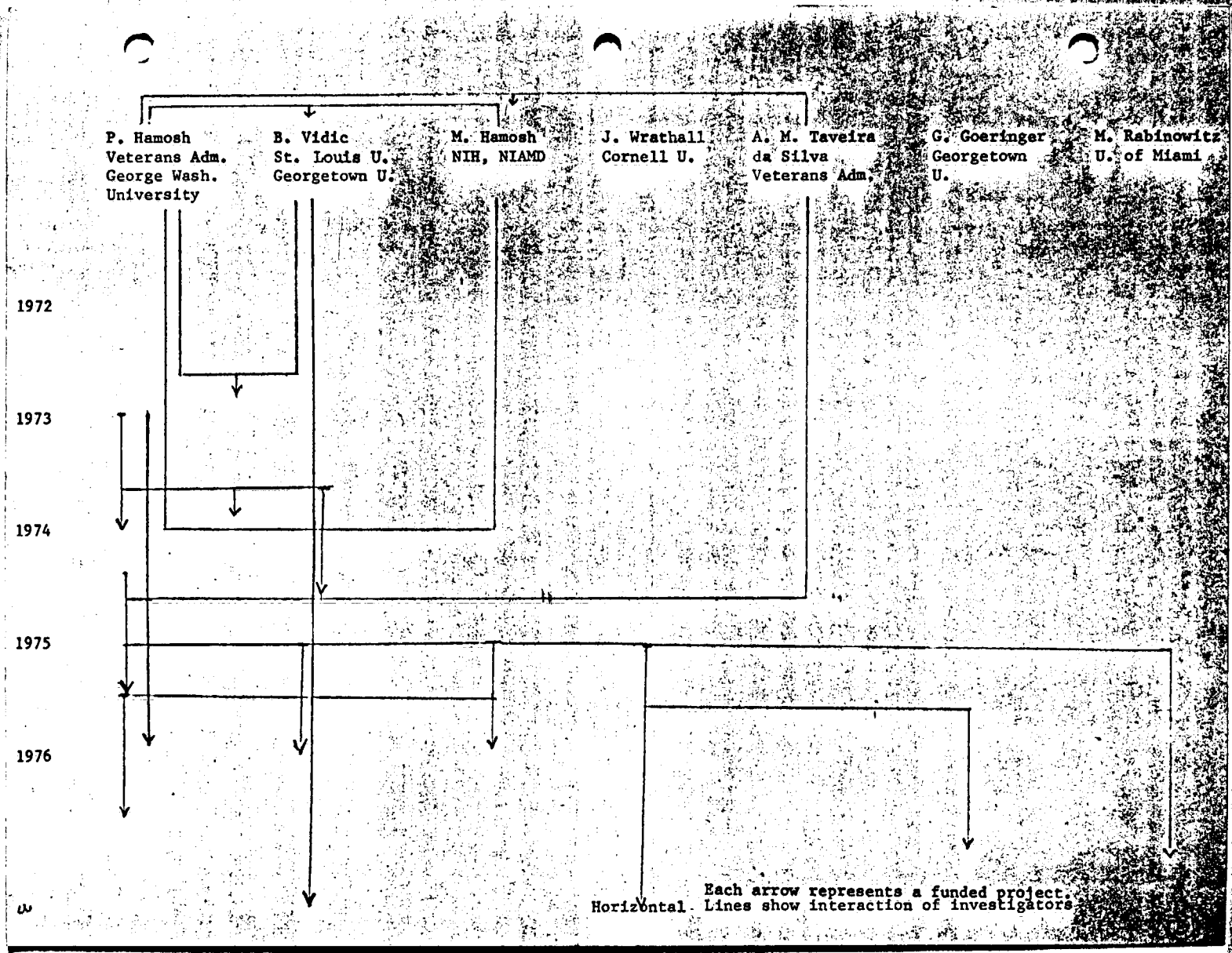

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Achievements of the Investigators from 1972.

Since the inception of the groups' activities, P. Hamosh's main thrust was the establishment of the framework for a major interdisciplinary effort in lung research. The goals were to 1) establish a laboratory in respiratory physiology 2) find a good structural investigator 3) attract a good biochemist who will establish a laboratory in lung biochemistry and 4) attract funds for research.

The first goal was achieved by maintaining the support of the Veterans Administration and keeping the equipment bought in the years 1970-1972. This led to completion of projects and three publications:

1. The Effect of Smoking a Single Cigarette on the Samll Airways. J. Appl. Physiol. 34:361, 1973.
2. Supine Hypoxemia and Erythrocytosis due to Air-Way Closure at Low Lung Volumes. Am. J. Med. 55: 80, 1973.
3. Air Bolus Method Compared to Simple Breath Method for Determination of Closing Volume. Am. Rev. Resp. Dis. 110: 518, 1974.

All this work was performed partially in the Veterans Administration and with the participation of Dr. Taveira Da Silva, who was trained by P. Hamosh. We also continued work on shearing stress which led to the presentation of the work in Cincinnati at the American Thoracic Society's annual meeting (The Effect of Shearing Stress on the Bronchial Mucosa. Am.. Rev. Resp. Dis. 109:694, 1974. and the manuscript (submitted in its present form to the CTR with a preliminary proposal). The human studies (Smoking and the "Small Airways") received the support of the CTR beginning January 1973 and has been pursued ever since. The arrival of Dr. Taveira Da Silva in late 1974 gave a tremendous impetus to this work. We are in the process of summarizing the results of the first two years of this research. We have asked basically two questions: Can one show after acute smoking ("provocation test") different responses in subjects, who might then be "reactors" or "non-reactors" to acute inhalation of cigarette smoke? The second question was more specific: what test to use for this discrimination? In the course of this research we produced data which are now being assembled into three manuscripts:

A. "The time course of response to smoking a single cigarette." We found that 1) response to smoke inhalation is maximal after just three puffs and remains plateaued irrespective of the amount of further smoking. 2) There is a quicker response of the large airways (airway resistance) and a

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slower in the "small airways" (maximum expiratory flow at low lung volumes) which is also more persistant in the latter 3) the response is individual and a "responder" will respond always with the same quantitative change.
B. "The most sensitive test for differentiating smokers from non-smokers: the MEF_{50}."

We have compared in a large series of young subjects all accepted pulmonary function tests and found only the MEF_{50} useful in differentiating with acceptable probability ($P < 0.02$). We used a great number of manipulations of the curve (MEF_{50}, MEF_{25}, MMFR, MTF_{50-75} and MTF_{75-85} etc.) and compared also water spirometer to dry spirometer to body plethysemsgraph. This latter part of work will probably be published separately.
C. "The effect of smoking a cigarette on the Helium Expiratory Curve". This work is essentially a study on the location of the equal pressure point (EPP) and deals with the mechanism of action. The results are negative and cast some doubt on the value of the helium curve.
At the time of writing we are engaged in two experimental protocols: 1) Racial differences in response. Recent work shows that in blacks it is difficult to separate smokers from non-smokers on the basis of pulmonary function tests. We found that their acute response to cigarette smoke does not differ from the whites. This is a very important finding and we are in the process of collecting enough data to achieve the level of statistical significance necessary for publication.
2) We have done work on using different Kentucky brand Research cigarettes and comparing the response. The response is definitely dose dependent (1R1 is higher that 1A1). 3) we are about to start to study the effect of smoking a cigarette on regional lung function using ^{133}Xe.

We hope to go on with this research within the framework of the program project after 1975 (project #1D).

In January 1973 we received a grant from the Washington Heart Association for the investigation of the effect of ventilation of lung surfactant metabolism. This was followed by a second award with Dr. Vidic as Principal Investigator in July 1973 and a third in January 1975. These grants were the initial resources on which cooperation between P. Hamosh, B. Vidic and M. Hamosh was based. M. Hamosh started working with us on weekends, when still at NIH. The project was essentially the study of the incorporation of ^{3}H palmitate into isolated pefused lungs ventilated at different rates and tidal volumes. This work was presented at the meeting of the eastern section

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of the American Thoracic Society in Washington, D.C., 1974. (The effect of Ventilation on ^{3}H-palmitate uptake by isolated perfused rat lungs. Proc. A. T. S. 1:3, 1974), and some other aspects of it at the annual meeting of Anatomists in San Diego in 1974 (same title, J. Cell Biol. 63:359a, 1974).

P. Hamosh provided the physiology, while Dr. Vidic the autoradiography and Dr. M. Hamosh the biochemistry. This work will also provide the substance of Mr. Michael Blunda's Ph.D. thesis, who was trained by Dr. P. Hamosh.

Using the resources from above grant and in collaboration with Drs. Yeager (from the other Program Project) and Dr. Schechter, the hormonal control of lipoprotein lipase in lung was investigated in rats. This manuscript is now in preparation. Other work done on lipoprotein lipases was all in the framework of this preliminary studies which were fleshed out with the beginning of additional support by the CTR "Smoking and Lung Metabolism: awarded to Drs. Vidic and Hamosh in January 1974. Therefore, during 1973 the second goal was achieved by the inception of cooperation between Drs. Vidic and P. Hamosh. The third goal was achieved in June 1974 when Dr. M. Hamosh joined full time the Department of Anatomy at Georgetown University and became a full partner in the group. Most of the work mentioned above will be described in detail in her narrative, since she is the principal author.

In 1975 two more grants from the Washington Heart Association are supporting the work on Lipoprotein Lipase. One was awarded to Dr. M. Hamosh as principal investigator to pursue the finding on the effect of estrogen in the rat and apply them to humans. The other was awarded to Dr. Paul Hamosh to test his hypothesis of the possible role of chylomicrons in atherosclerosis. This latter work combines the research on vascular shearing stress with the research on lipoprotein lipase. It is worthwhile to mention that the Hamoshes collaborated successfully already in the early sixties (J. Clin. Invest. 42: 1648, 1963).

Collaborative work between P. Hamosh and Vidic has led to a number of projects in progress. Most of these are centered around the morphology of surfactant (see manuscript submitted with progress report 936R2).

P. Hamosh has also done cardiovascular work during this time, mostly with the assistance of A. da Silva. Thanks to a grant from the Washington Heart Association, the effect of volume loading on lung compliance was studied. This experimental design was also used to complete a study on the mechanism of hepatojugular reflux which was submitted for publication. With Dr.Kulczycki from Children's Hospital, work was done on children with cystic

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fibrosis and the results were published in the Cystic Fibrosis abstracts (1974). In cooperation with Dr. Ungerleider for the National Biomedical Research Foundation a computer program was worked out for automated assessment of emphysema. With Dr. Da Silva, a drug study was completed on the effect of prostaglandin $F_{2\beta}$ on the airways (submitted to "Prostalgandins" for publication). A computerized ventilation performance scan on cancer patients is in progress funded by a contract with the Veterans Administration.

All these activities led to the achievement of the fourth goal; attracting funds for a relatively large scale operation. The new goal is to expand and unify these activities under one single effort toward which goal the program project was directed.

M. Hamosh

Early work on fatty acid release from the liver (1961) and on the hormonal regulation of fatty acid release from adipose tissue, (1960) led to the first joint study of M. and P. Hamosh on lipid metabolism in human adipose tissue (1963). This was one of the first studies in the human and showed that there are marked differences in fatty acid metabolism between the "internal" (omental, perirenal) and subcutaneous adipose tissues. The metabolism of adipose tissue was further pursued by M. Hamosh with special emphasis on changes leading to the development of obesity (1964). In 1965 M. Hamosh started a two year sabbatical at the NIH; she decided to broaden her experience and to spend this time in studies related to protein and nucleic acid metabolism. The result of these studies was the discovery of enhanced protein synthesis in a cell-free system prepared from skeletal muscle undergoing rapidly compensatory hypertrophy (Publications: 1967, 1968). The years 1968-1974 were spent at the NIH. During this period the major interest was in the regulation of lipid transport and the digestion of dietary fat. The uptake of circulating lipid (which is present in the blood chiefly as triglyceride in chylomicrons and very low density lipoprotein) is catalyzed and regulated by the enzyme lipoprotein lipase. Research was therefore centered around this enzyme, with the specific objective to elucidate the relationship between the lipemia of pregnancy and the activity of this enzyme. The major findings were that the hormone prolactin induced changes in the level of lipoprotein lipase activity in adipose tissue (inhibition) and mammary gland (stimulation). These changes in enzyme activity channel dietary triglyceride from storage in adipose tissue to milk formation in the mammary gland (see references). The presence or absence of lipoprotein

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lipase in the milk of the lactating animal can be taken as a measurement of holocrine or apocrine secretion (see reference 1971).

The mechanism of LPL activity was studied in the isolated, perfused adipose tissue and mammary gland, by biochemical and especially developed electronmicroscopic cytochemical methods Studies on the digestion of dietary triglyceride were initiated when it became apparent that the low pancreatic lipase activity of the newborn is insufficient to digest the large amounts of fat present in milk. These studies led to the discovery of a new digestive enzyme, that is secreted from lingual serous glands (Von Ebner) and is active in the stomach in the digestion of dietary TG to partial glycerides and FFA: A similar digestive enzyme has subsequently been described in the human. EM and biochemical studies conducted jointly with Dr. A. R. Hand are now in progress on the development of secretory activity in rat lingual serous glands These studies show that secretory activity (measured by the present of lingual lipase) can be detected 2 days before birth in the rat. At this age the lingual serous glands are not yet developed, whereas the mucous glands are well developed. In the suckling and adult rat, lingual lipase is secreted by serous glands, it seems therefore, that during early development the enzyme is secreted by demilune cells (serous cells present within mucous secreting glands).

Accomplishments since 1973 with P. Hamosh.

In 1973 M. Hamosh was invited by P. Hamosh to help set up the methodology for biochemical studies of the lung. These studies can be divided into 3 parts:

1. The study of lung metabolism in the isolated perfused lung.
2. Lipoprotein lipase regulation of activity and role in lung metabolism.
3. The effect of smoking on lung metabolism - to be studied in the following tissue preparations: isolated perfused rat lung; lung slices and isolated cultured type II alveolar cells.

The questions which we want to answer using the isolated perfused lung are:

a. what is the relationship between ventilation and the synthesis (and possible use) of surfactant.
b. Are there changes in the synthesis and secretion of surfactant as a function of vascular perfusion.
c. Does exposure to air pollutants (i.e.: cigarette smoke) change the ability of the lung to synthesize surfactants (at normal ventilation or during hyperventilation).

The first part (a) of the study has been completed and theresults were presented by Dr. M. Hamosh at the American Thoracic Society Meeting in October 1974. Briefly, we have found that ventilation at high tidal

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volume (40% of TLC) doubles the uptake of infused ^{3}H-palmitic acid and its incorporation into phosphatydil-choline. Thus palmitate incorporation into phospholipids (surfactant) is a function of respiration. Autoradiography of the perfused lung shows a faster flux of the label from endoplasmic reticulum through lamellar bodies and onto the alveolar surface at high tidal volume (TV) ventilation. Only in the high tidal volume ventilated lung does one see silver grains associated with myeline figures (presumably "used" surfactant) in the alveolar space (ref. 1974). We are now gathering data on part b. of this topic, namely we study the effect of changes in the vascular perfusion on the incorporation of ^{3}H-palmitate into lung phospholipids. We plan to start the last part of this project (c) very soon, and will start smoking experiments as soon as we obtain full data on our metabolic studies in smoking rat that are now in progress.

Lipoprotein Lipase

Although a great number of studies have focused on the role of free fatty acids, especially palmitic acid in lung metabolism, the contribution of triglyceride-fatty acids (the major reservoir of fatty acids) has not yet been investigated. As indicated above, uptake of blood triglyceride-fatty acids involves hydrolysis of the triglyceride to free fatty acids and glycerol; this hydrolysis is catalyzed and regulated by lipoprotein ipase.

We have shown that lung has a high lipoprotein lipase activity; we have also shown that infusion of heparin into isolated perfused rat lungs causes the immediate release of the enzyme, indicating its presence in or near the capillary wall. These findings indicate that the lung is able to utilize blood triglyceride. In addition we have shown that contrary to the enzyme in other tissues, enzyme activity in the lung remained high during fasting 8-72 hours. This indicates that, during food deprivation the lung and heart are the only tissues able to utilize circulating triglyceride-fatty acids. This work was presented at the annual meeting of the American Physiological Society, Atlantic City, 1974, followed by publication in Biochim. Biophys. Acta 380: 132, 1975. We have pursued the study of lipoprotein lipase with special emphasis on the hormonal regulation of enzyme activity in the lung.

Our studies have shown that the lung maintains a high level of lipoprotein lipase activity under physiological and pathological conditions known to decrease enzyme activity in other tissues. The manuscript of this study is now in preparation. In the course of this study we made the interesting observation that estrogen administration to male or ovariectomized female rats markedly decreased lipoprotein lipase activity in adipose tissue. We think,

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therefore, that the hyperlipemia which accompanies high plasma estrogen levels (such as pregnancy or contraceptive therapy) could be due to the marked inhibition of adipose tissue lipoprotein lipase activity. This work was presented at the meeting of the American Federation for Clinical Research, New Orleans, La., January, 1975, and subsequently published in J. Clin. Invest. 55: 1132, 1975. We plan to continue these studies on the relationship between high estrogen levels, hyperlipemia and adipose tissue lipoprotein lipase activity in the human (on a Washington Heart Association Grant awarded to M. Hamosh).

3. The Effect of Smoking on Lung Metabolism

a. As described above, our studies on the effect of smoking on the incorporation of palmitate into the isolated perfuse, ventilated and especially hyperventilated lung will be initiated as soon as our studies on the normal lung are completed.

b. Lung slices- In preparation for our studies on the effect of tobacco smoke on lung metabolism we have first investigated lung metabolism in normal rats. Since there is in general a lack of standardization of the methodology employed in the study of lung metabolism we have adapted the following methods to the study of lung metabolism in tissue slices:

1. Glucose Metabolism measured as the amount of $U^{14}C$ Glucose metabolized to $^{14}CO_2$ (aerobic metabolism), lactate (glycolysis) and incorporated into tissue lipid.

2. Protein Synthesis measured by the incorporation of leucine into lung protein.

3. Glucoprotein Synthesis measured by the incorporation of glucosamine.

4. Lipid and Phospholipid Synthesis measured by the uptake and esterification of labeled palmitate.

We have chosen the tissue slice method since it is the least time consuming and according to recent studies has an identical glucose metabolism as that of the perfused lung.

We have adapted the following analytical methods to the study of lung composition:

RNA, DNA, protein, lipid analysis (separation, identification and quantitation of the individual components of the major lipid classes-phospholipids and neutral glycerides).

c. Isolated type II alveolar cells by a method based on a modification of the methods of Rounds and Mason. The type two alveolar cells maintain intact, concentric lamellar bodies after isolation by gradient centrifugation. We have succeeded in culturing these cells, but at present we are worried about the loss of the typical structure of the lamellar bodies in these cultured cells. The isolated cultured cells show a corticosteroid stimulated incorporation of ^{3}H palmitic acid into lecithin.

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Jean R. Wrathall

From 1970 to 1974 research activites centered on the control of the expression of differentiated functions and malignant potential in cultured cells. The model system used consisted of a clone (B559) of cells derived from the B16 mouse melanoma. These cultured cells express the "normal" attributes of the "tissue" of origin.. They synthesize melanin through the production of the enzyme tyrosinase which is packaged in specialized cell organelles, melanosomes. The ultrastructure and manner of formation of the melanosomes appear identical to that of mouse melanoma cells, or normal melanocytes in vivo. Cells of clone B559 also retain their malignant potential as shown by tumor formation upon injection into syngeneic C57BL/6 mice. To probe the control of these cell functions we have been using the thymidine analogue 5-bromodeoxy - uridine which has been shown to preferentially and reversibly suppress cell-specific functions in a variety of cell types. In the melanoma cells both differentiated function (melanogenesis) and malignancy (tumorigenicity) are suppressed by concentrations of BrdU that do not significantly inhibit overall cell metabolism or growth. Besides demonstrating that malignant potential is reversible and may be regulated in a manner similar to the regulation of normal cell-specific functions, our studies have opened the way to investigating the significance of various factors (growth potential, immunogenicity, tumor viruses, fibrinolytic activity) in the overall "malignant potential" of tumor cells. Copies of representative publications (including a review article) from these investigations are included).

Since joining the Department of Anatomy of Georgetown University in January, I have been: a) establishing a laboratory in which cell tissue organ culture experiments may be successfully performed; b) continuing my research with the melanoma cell system; and c) expanding my research interests to include the regulation of lung cell functions. My present interest in lung cells has resulted from interactions with a number of Georgetown researchers involved in pulmonary research, especially Dr. B. Vidic and M. Hamosh of my department and Dr. P. Hamosh of the Physiology department. I have become convinced that study of the functions of lung cell types at the cellular level through cell culture techniques would yield worthwhile information especially as part of a integrated research program encompassing pulmonary physiology, biochemistry, development and ultra-micro-anatomy.

I have already become involved in directing research activities of Ms. Susan Compton, a doctoral candidate in this department in her studies of the function of aveolar type II epithelial cells in culture. This project is far from complete, but considerable progress has been made in the last several months.

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The primary aim has been to define conditions for the mass cultures of type II cells such that they continue to produce surfactant in vitro. To this end a number of variables have been investigated. At present the best success has been obtained by isolating type II cells from 3-4 day old neonatal rats by the procedure of Rounds (personnal communication) and establishing the cells as monolayer cultures with medium 199 with 10% fetal calf serum and dexamethasone (0.5 µg/ml). After 28 hours in culture the cells exhibit the characteristic lamellar bodies of type II cells in vivo. Autoradiographic studies have been initiated to establish the incorporation of 3H-palmitate into lamellar bodies per se, but these have not yet been completed. However, the growing cultures actively incorporate 3H-palmitate lecithin after culture for at least 3 weeks. Cells examined at 3 weeks of culture do not contain typical lamellar bodies but a high proportion have lipid-like inclusion bodies. The nature of these bodies and the time course of their appearance during culture are currently being studied.

To facilitate further studies of the culture conditons that would permit production of normal lamellar bodies in vitro we are now concentrating on light microscopic histochemical techniques for phospholipids that can be used to monitor more easily the effects of altered conditions on the cell populations.

As stated in Project 3 of the Program Project, I will also derive cultures of type II cells by the clonal isolation technique that has already been used for this purpose. However, if Ms. Compton's investigations succeed in establishing a procedure for obtaining mass cultures of type II cells these would be used for our experiments, as the clonal technique always carries the risk of selecting a clonal population that is aberrant in some respect as compared to the whole population in vivo.

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